ANALYSIS OF THE KINETICS AND REGULATION OF CYTOKINE GENE EXPRESSION DURING THE PRIMARY IN VIVO IMMUNE RESPONSE TO KILLED BRUCELLA ABORTUS

1992

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ABSTRACT

Title of Thesis:

Analysis of the Kinetics and Regulation of Cytokine Gene Expression during the Primary *in vivo* Immune Response to Killed *Brucella abortus*

Yichun Jian; Candidate, Masters of Science, 1992

Thesis directed by: William C. Gause, Ph.D., Assistant Professor,

Department of Microbiology

Cytokines are thought to be important mediators of effector lymphoid cell function, but their expression during an *in vivo* immune response has not been well documented. Based on *in vitro* studies, immunization of mice with *Brucella abortus* (BA) has previously been characterized as a Th1 response, associated with elevated IL-2 and IFN-γ (Th1 cytokines) and depressed IL-4 and IL-5 (Th2 cytokines). In this study we attempted to determine: 1) the Th1/Th2 cytokine gene expression pattern following primary immunization of mice with BA; 2) whether cytokines elevated early in the response influence the later cytokine gene expression pattern; and 3) whether $CD4^+$ T cells or other cell types might contribute to the expected elevation in Th1 cytokines. Total RNA was purified from the spleens of mice 1 hour and 1 to 7 days after BA

immunization. A quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to evaluate the expression of seven cytokine genes, all of which encode cytokines that are secreted by T cells and have been found to be specifically associated with the Th1 or the Th2 response. These were IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, and IL-10. IL-6 and IL-10 were elevated by 1 hour and IFN-γ was elevated by 1 day after BA immunization. Although IL-6 was reduced to baseline levels by 1-2 days, IFN-γ and IL-10 remained elevated throughout the time period examined. Similar results were obtained following BA administration in the footpads of mice and analysis of cytokine gene expression in the draining popliteal lymph node, except that the elevation of IL-6 was more prolonged. Cell sorting analysis of spleen cells from BA-immunized mice revealed that although at 1 hour non-T cells were responsible for the increased cytokine expression, by 1 day, Thy-1$^+$ cells were the primary source of elevated IL-10 and IFN-γ, and that CD4$^+$ cells expressed elevated IL-10, but not IFN-γ. Anti-IFN antibodies administered one day before BA immunization altered the cytokine gene expression pattern at four days after BA immunization, with elevations occurring in IL-4, IL-5, and IL-9, but little change occurring in IFN-γ or IL-10. In contrast, administration of anti-IL-2 antibodies did not affect the cytokine gene expression pattern. These data suggest that a unique IL-2 independent pattern of Th1/Th2 cytokine gene expression, which is maintained throughout the primary response, manifests itself by 1 day after BA immunization. The source of the elevated cytokines includes CD4$^+$ and CD4$^-$ Thy-1$^+$ cells and the consequent pattern is maintained in part by elevated IFN-γ.

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By

Yichun Jian

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ABBREVIATION

Ab: antibody

Ag: antigen

APC: antigen presenting cells

BA: *Brucella Abortus*

Con A: conconavalin A

DTH: delayed-type hypersensitivity

FACS: fluorescent activated cell sorter

GaMδ: goat anti-mouse IgD antibody

Ig: immunoglobulin

IL: interleukin

IFN: interferon

LPS: lipopolysaccharide

MACS: magnetic activated cell sorter

MHC: major histocompatability complex

NK: natural killer cells

PMN: polymorphonuclear cells

RT-PCR: reverse transcriptase-polymerase chain reaction

Th1: T helper 1 cells

Th2: T helper 2 cells

Thp: T helper precursor cells or virgin T helper cells

TNF: tumor necrosis factor

INTRODUCTION

TH1 /TH2 CELL POPULATIONS

1. Brief History and Characteristics

Lymphocytes were first divided in 1968 into two classes: T lymphocytes, which are important in delayed-type hypersensitivity (DTH), cytotoxicity and regulation of the immune system, and B lymphocytes, which are responsible for antibody (Ab) production (1,2). Subsequently, T lymphocytes were further subdivided into $CD4^+$ and $CD8^+$ T cells by the subset-specific surface Ag CD4 and CD8. $CD4^+$ T cells are Class II MHC-restricted and $CD8^+$ T cells are Class I MHC-restricted (1). Although originally defined as T helper (CD4) and T cytotoxic/suppressor cells (CD8), because of considerable overlap in these functions this distinction is no longer useful. However as $CD4^+$ cells are referred to as T helper cells since they were originally found to provide help for B cell activation and differentiation (3). $CD8^+$ cells are also called cytotoxic T lymphocytes (CTL) since they were originally found to be able to kill virus-infected cells (3). Several studies have indicated that the $CD4^+$ T cell population can be further subdivided into at least two populations (4-9) based on functional criteria. In 1986, Mosmann *et al.* (10) identified two types of murine $CD4^+$ T cell clones from immunized mice, according to the profiles of lymphokines secreted upon alloantigen or Con A restimulation *in vitro*. One subset, called the Th1 phenotype, was defined by its ability to secrete IL-2, IFN-γ and lymphotoxin. Later this subset was shown to be associated with DTH, cytotoxicity and IgG2a class switching (10-15). The other subset, the Th2

phenotype, secreted IL-4, IL-5, IL-6, IL-9, IL-10, P600 and was shown to be associated with immediate hypersensitivity, IgE and IgG1 isotype switching (10,12,14-17). Unlike the other lymphocyte classes, no surface Ag has yet been found to be specific for either Th1 or Th2 cells.

2. Significance

The classification of lymphocytes is obviously important in helping to understand the immune system and the immune response. The distinctive patterns of lymphokine synthesis by Th1 versus Th2 cells and the associated different helper functions of Th subsets demonstrated the significance of the Th1/Th2 model. But the generality of this Th1/Th2 thesis *in vivo* has been controversial since its discovery, particularly because much of the original data was derived from long-term cultured T cell clones (10,18). Recent studies of immunity to infectious agents, especially many parasites like *Leishmania (19-23), Heligmosomoides polygyrus (19,20,24), Nippostrongylus brasiliensis (19,20,25)*, and *Schistosoma mansoni* (19,20), have documented patterns of immunoglobulin isotype production and cytokine production characteristic of a Th1-like or Th2-like response, suggesting this dichotomy occurs *in vivo* and that it is important physiologically, since with particular pathogens a Th1 or Th2 response can be either host protective or associated with disease progression. As yet, an actual Th1 or Th2 cell has not been identified *in vivo*, a task that would be quite difficult because it would require measurement of cytokine production from single cells stimulated *in vivo*.

An example of pathogens evoking a Th1 or Th2 response is provided in the

extensively studied mouse model of *Leishmania major* infection (21). Here, BALB/c mice exhibit a Th2-like response that is associated with severe, generalized disease, and these animals exhibit high IL-4 and IgE antibody levels, and low IFN-γ and DTH reaction levels. On the other hand, C57BL/6 mice produce a Th1-like response that is associated with a contained, local infection and cure, and these mice can be characterized by low IL-4 and IgE antibody levels, high IFN-γ levels, and strong DTH reactions. Treatment of C57BL/6 mice with anti-IFN-γ results in a Th2-like response associated with disease progression as observed in the BALB/c strain (22).

Whereas the Th1/Th2 pattern is well-documented in mice, the relevance of Th1/Th2 cells to humans been has been only recently examined. Studies from several laboratories where individuals were examined with active allergy or infection suggest that functional subsets of $CD4^+$ T cells do exist in man (26). The work of Romagnani *et al.* (27-29) showed that T cell clones from normal and atopic individuals responsive to tuberculin purified protein derivative (PPD), or to parasite antigens, could be classified into functional subsets of $CD4^+$ T cell clones analogous to those in the mouse. Similar results have been seen in studies of leprosy patients, showing that IL-2, IFN-γ and LT were abundant in tuberculoid lesions, indicating a host protective response, whereas IL-4, IL-5 and IL-10 were characteristic of lepromatous lesions, associated with disease progression (30,31).

The outcome of the immune effector cell function can to some extent be predicted simply from the cytokines produced in a given immune response. On the basis of many *in vitro* and *in vivo* studies, it is now believed that the cytokines associated with a Th1

or Th2 response regulate immune effector cell functions. Allergic or immediate hypersensitivity responses are mediated by Th2 cytokines. This is accomplished by their ability to secrete IL-4, which stimulates Ig class switching to IgE (32,33); IL-3, IL-4, IL-9, and IL-10, which can promote mast cell growth (34-38); and IL-5, which stimulates eosinophil growth (39). Inflammatory reactions with the features of a Jones-Mote type of DTH are mediated by Th1-type immunity (40,41). IFN-γ, which can activate macrophages and promote their function, was demonstrated to play a primary role in this response (41). All these studies revealed that T helper lymphocytes and the cytokines they produce play a crucial role in determining the outcome of infection in terms of both protective immunity and immunopathology.

OTHER T HELPER CELL SUBSETS AND THEIR RELATIONSHIP WITH TH1/TH2 CELLS

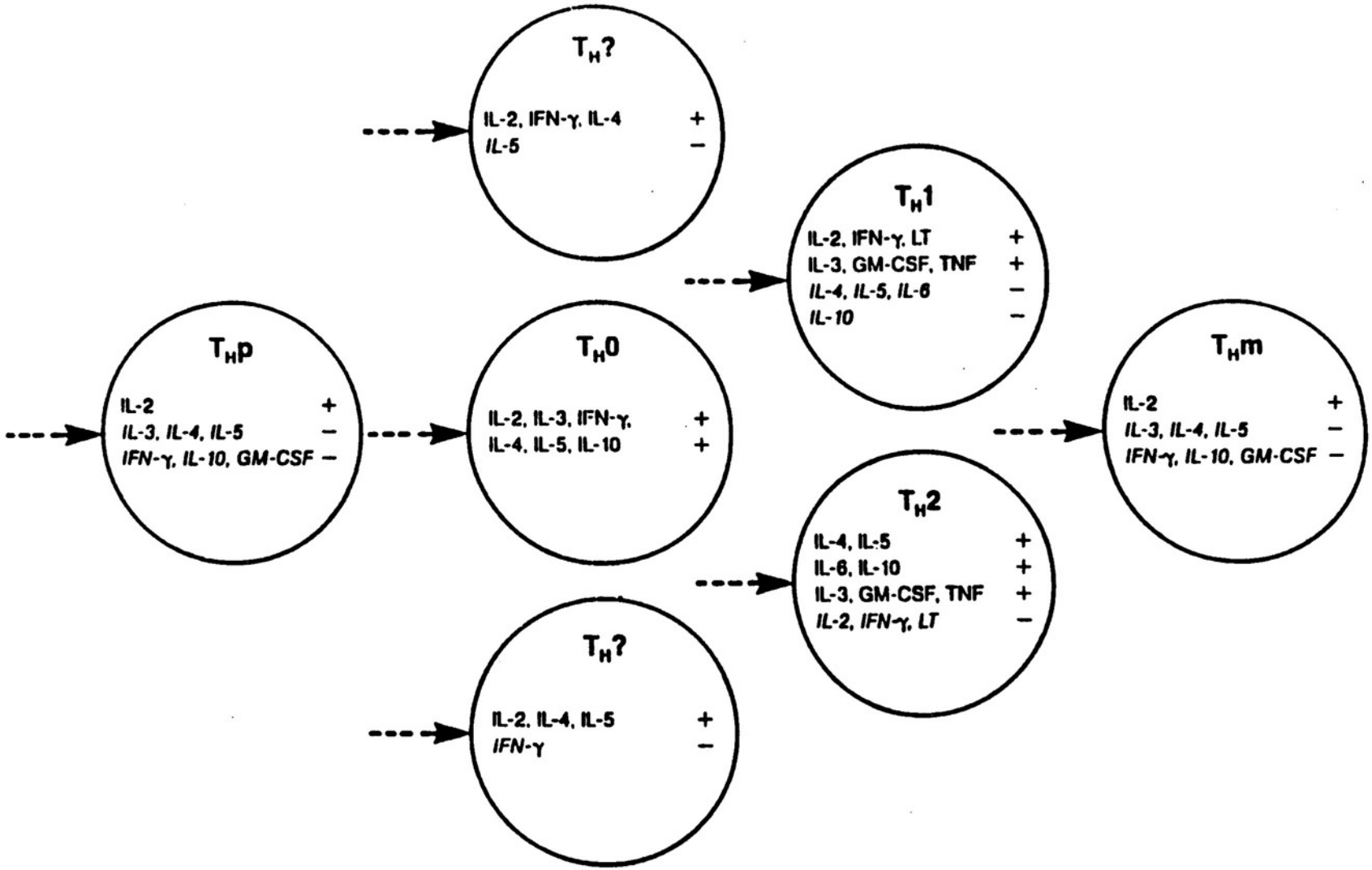
The Th1 and Th2 cytokine patterns are well-defined and stable, at least in tissue culture, and many mouse T cell clones fit either the Th1 or the Th2 cell pattern (42). However, other cytokine secretion patterns can be readily observed among mouse T cell clones and many human T cell clones (26,43-48). Naive T cells (Th precursor cells), when first stimulated in *vitro*, produce large amounts of IL-2 but not other T cell cytokines (with the possible exception of the recently cloned IL-9), and acquire the ability to produce other cytokines such as IL-4 and IFN-γ a few days later (43,49,50). Th precursor cells (Thp) and other T helper cell clones, with a cytokine synthesis pattern different from the Th1 and /or Th2 patterns, are often derived from short-term stimulated

Th cells. It has been hypothesized that precursor T cells (Thp), when stimulated by interaction with antigen presenting cells (APCs) presenting MHC + foreign peptide, initially produce IL-2 and, in the presence of this cytokine, proliferate (43,49-51). The resulting milieu favors the development of the so-called Th0 cells, which produce a mixture of virtually all possible Th1/Th2 cytokines including IL-2, IFN-γ, IL-4, IL-5, IL-6 and IL-10 (18,43). If the response is chronic, the more defined Th1 and Th2 patterns develop, with a Th1 response being associated with delayed type hypersensitivity, elevated IFN-γ and IL-2, and in the mouse increased IgG2a (11,40), and a Th2 response being associated with immediate hypersensitivity, elevated IL-4, IL-5, IL-6, IL-9 and IL-10, and increased IgG1 and IgE (12,14,15,17,40). The current hypothesis about the diversity of Th cells and their delineation is shown in Fig.1 (42). Although the types of Th cells have been quite well documented *in vitro*, the differentiation pathways involved and their roles are not clear.

ROLE OF HELPER T CELLS IN PRIMARY IMMUNE RESPONSE

The T cell is a key factor in the activation of the primary immune response. How it is activated is critical since the T cell controls the subsequent outcome of the response. At early stages of the response, APCs are initially stimulated by foreign Ag, probably conserved constituents of pathogenic organisms, such as lipopolysaccharide (LPS) or double-stranded RNA (52). Macrophages quickly differentiate into effector cells which can mediate a non-antigen-specific host-protective response (innate immunity) prior to

Figure 1. Cytokine secretion phenotypes of helper T ($CD4^+$) cells. The proposed relationships among the various cytokine secretion phenotypes are shown by dotted arrows since there is no clear evidence for the pathways involved. This figure is adapted from Street and Mosmann (42).


T_H?
IL-2, IFN-γ, IL-4 +
IL-5 −
T_H1
IL-2, IFN-γ, LT +
IL-3, GM-CSF, TNF +
IL-4, IL-5, IL-6 −
IL-10 −
T_Hp
IL-2 +
IL-3, IL-4, IL-5 −
IFN-γ, IL-10, GM-CSF −
T_H0
IL-2, IL-3, IFN-γ, +
IL-4, IL-5, IL-10 +
T_Hm
IL-2 +
IL-3, IL-4, IL-5 −
IFN-γ, IL-10, GM-CSF −
T_H2
IL-4, IL-5 +
IL-6, IL-10 +
IL-3, GM-CSF, TNF +
IL-2, IFN-γ, LT −
T_H?
IL-2, IL-4, IL-5 +
IFN-γ −
Virgin T_H cells
Short-term stimulated
Chronically stimulated
Long-term memory

the development of an antigen-specific response (acquired immunity). Before specific helper T cell clones are activated, endocytosed antigenic peptide must be processed and expressed in association with MHC class II on the surface of APC. This modified foreign Ag will be recognized by the T cell receptor (TCR), and in the presence of co-stimulatory signals, will result in the activation and differentiation of T helper cells into various functional subsets, which in turn will influence the development of the immune response (1).

IMPORTANCE OF CYTOKINES AND OTHER FACTORS IN THE INITIATION OF PRIMARY IMMUNE RESPONSE

T cell activation has always been a central step in the immune response. Extensive work has been accomplished on the regulation of effector cell functions by Th1/Th2 cytokines. However, how the T cells themselves are differentially activated during the primary immune response has not been extensively examined. Several factors may determine the choice of Th cell subsets. Following initial interaction of the immunogen with the host organism, macrophages and other accessory cells including mast cells and natural killer cells can be rapidly activated. The resulting milieu may consequently affect the differentiation of T cells during antigen presentation by the APC. The nature of the APC is likely to have a profound influence on the type of functional T-cell subset selected (53). The differences in APCs may exist in the enzymatic machinery required to digest protein antigens to produce peptides, such that different cell types may in fact present different epitopes. Different APCs may display differing

second signals, accessory surface molecules or secrete cytokines that influence the responses (53). Evidence already exists that macrophages and NK cells can produce IFN-α, IFN-β, IFN-γ, and prostaglandins early in an immune response (54-57), while B cells can produce IL-10 (58). Cytokines usually have a direct effect on the activation, differentiation and proliferation of T cells. In the generally accepted two-signal model of lymphocyte activation originally proposed by Peter Bretscher and Melvin Cohn (59), it was suggested that besides the interaction between TCR and Ag/MHC, an accessary second signal, such as an interleukin, is necessary for the activation of T lymphocytes. Besides acting as secondary signals for T cell activation, some cytokines function to direct T cell differentiation and also can act as growth factors for the proliferation of the already differentiated T cells. Recent evidence shows that the cytokine environment during T cell activation has a great influence on the type of Th cell that will be generated (60-63). Gajewski et al. (60) have shown that Th1 cells are preferentially obtained when $CD4^+$ cells are cloned in the presence of IFN-γ. Conversely, the presence of IL-4 in the *in vitro* Th cell cultures enhances the development of the IL-4/IL-5-secreting phenotypes, while suppressing the development of the IL-2/IFN-γ-secreting phenotype (63,64). Although many *in vitro* and *in vivo* data suggest that IL-4 and IFN-γ are the regulatory signals that maintain the Th1 and the Th2 type immune response, respectively (18,20), only recently have some studies suggested that IL-4 can direct Th0 cells to differentiate into Th2 cells and block the differentiation of Th0 cells to Th1 cells (64).

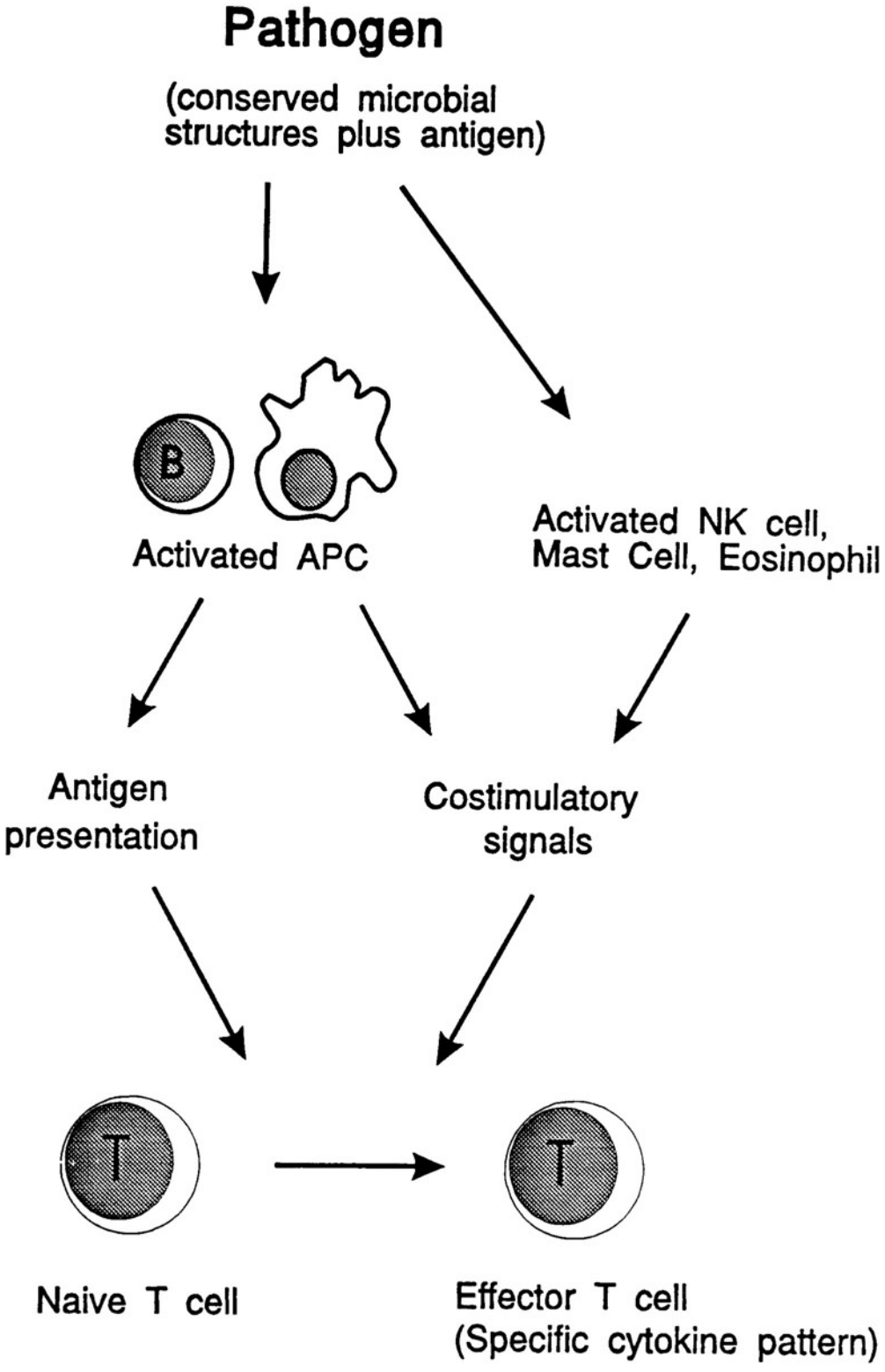
The source of the specific cytokines, such as IL-4, that initially influence the development of Th1 and Th2 cells can be non-T cells. It is known that mast cells and

eosinophils can be directly activated by some immunogens to release IL-4 and IL-5, respectively (65,66) and IFN-γ can be produced by activated natural killer cells (67). IFN-α/β, produced in large part by macrophages, is a potential candidate for the regulation of the IL-4/IFN-γ ratio (68). Recent studies have suggested that endogenously produced IFN-α/β can alter a predominantly Th2 response such that the ratio of IFN-γ to IL-4 gene expression is increased and serum IgG2a levels are elevated (69). Once a Th1 or Th2 cell subsets is committed, specific cytokines produced by that T cell subset will help maintain the Th1 or the Th2 response through both stimulatory and suppressive activities. The outline of T cell activation is shown in Fig. 2.

THE STUDY SYSTEM IN THE PROJECT

Brucella abortus (BA) is a facultative intracellular bacterial pathogen that is thought to stimulate a T-independent response initially, followed by a Th1-like response (43). Cell-mediated immunity plays a major role in the protective immunity, although it has been noted that some transient resistance to BA can be transferred passively in serum to mice (70). After gaining entry to the body, the organisms have been found to survive within the cells of the reticuloendothelial system, particularly within phagocytic cells of the immune system. Subsequently, they are transported to the lymph nodes, where macrophages and polymorphonuclear cells (PMNs) die, releasing more bacteria. In animals where the acute infection is not controlled, the bacteria become disseminated and eventually localize in the spleen and liver. Four phases of BA infection

Figure 2. Differential activation of T cells by specific pathogens. T cells activation requires two signals: antigen presentation and costimulatory signals such as cytokines. Depending on the nature of the signals, T cells will be activated differently.


Pathogen
(conserved microbial structures plus antigen)
B
Activated APC
Activated NK cell, Mast Cell, Eosinophil
Antigen presentation
Costimulatory signals
T
T
Naive T cell
Effector T cell (Specific cytokine pattern)

were shown in CBA/N mice inoculated by the intravenous route: (1) an early, active growth phase; (2) a bactericidal stage apparent in the liver at 7 to 21 d postinoculation and in the spleen 14 to 17 d postinoculation; (3) a plateau phase; and (4) a final recovery stage. If the organism can somehow resist activated macrophages, a chronic infection will be established (70).

Components of BA that contribute to the immunity against BA are under extensive investigation, among which lipopolysaccharide (LPS), is one of the most thoroughly studied. In contrast to many endotoxins, *Brucella* LPS is nonpyrogenic, does not induce a localized Shwartzman reaction, does not increase the susceptibility to histamine, does not activate complement to any significant level, and is a very weak mitogen for murine B cells (71). For lethality and interferon production, at least 10 times more *Brucella* LPS than enterobacterial LPS is needed and the ability of *Brucella* LPS to activate neutrophils is weaker as well (72-74). On the other hand, *Brucella* LPS, but not enterobacterial LPS, stimulates the production of antibodies (IgG and IgM), IL-1, and tumor necrosis factor in endotoxin-resistant C3H/HeJ mice (72,73,75). Recently BA has been considered as a potential vaccine carrier (71). Since it can stimulate human B cells even in the relative absence of T cells, it may be particularly useful in the treatment of infections that cause T cell defects, such as HIV. The weak endotoxic activity of BA also makes it attractive as a potential vaccine carrier (71).

The bacterial immunogen, *Brucella abortus* (BA), has recently been extensively examined as a model system for the induction of a Th1 response in mice. Killed, fixed BA were found to stimulate IFN secretion and a predominantly IgG2a response which

could be suppressed by the administration of anti-IFN-γ antibodies (11). Street *et al.* (43) demonstrated that long term T cell clones derived from T cells isolated from the spleen 8 days following a primary BA immunization were exclusively of a Th1 phenotype (as defined by elevated IL-2 and IFN-γ), while clones isolated from the spleens of mice given a primary infection with a helminth (*Nippostrongylus brasiliensis*) were primarily of a Th2 phenotype, as defined by elevated IL-4 and IL-5. Also freshly isolated $CD8^-$ spleen cells from BA immunized mice exhibit markedly increased IFN-γ production but little increase in IL-4 or IL-5 following restimulation *in vitro* with Conconavalin A (Con A) (43).

GOAL OF THESE STUDIES

The general goal of this project is to elucidate the role of cytokines in the initiation of the *in vivo* primary immune response that precedes a Th1 response. The primary immune response is little understood both with respect to the cell populations involved and to the pattern of cytokine production resulting from antigen stimulation. While processed antigen bound to MHC stimulates T cells via the TCR resulting in the activation of specific clones, additional signals, including cytokines, may regulate T cell differentiation, influencing which cytokines T cells secrete. These signals may be produced by the APCs and also other accessory cells including natural killer and mast cells which are frequently activated prior to the T cells, since they can be rapidly stimulated by conserved structures characteristic of particular pathogens (52). These resultant signals, associated with certain pathogens, may thus influence the subsequent

T cell-mediated response, contributing to the development of a Th1 or a Th2 response.

In this study a killed and fixed gram negative bacterium, BA, was used as the immunogen. Previous studies, primarily based on *in vitro* analyses, have suggested that this bacterium elicits a strong Th1-like response (11,43). To examine at what stage a Th1 cytokine pattern is first identifiable *in vivo*, we utilized a highly sensitive quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay to measure changes in Th1 and Th2 cytokine gene expression during the course of the primary response. The time course for the analysis of cytokine gene expression included measurements at 1 hour and 1-7 days after i.v. injection of BA. Seven days was chosen as the end point since by 7 days, the response is well-developed with high levels of serum IgG2a and unchanged levels of IgG1 and IgE, consistent with the development of a Th1-like response.

We also were interested in determining whether particular cytokines increased early in the response influenced cytokine production at later stages of the primary response. An alternative hypothesis would be a fixed pattern of cytokine gene expression following BA administration that would not be influenced by the cytokine milieu. To distinguish between these two possibilities, we administered anti-cytokine antibodies to BA-immunized mice at levels that had been previously shown to be effective in other *in vivo* systems. Changes in both Th1 and Th2 cytokine gene expression were then monitored and compared to mice administered BA plus irrelevant Ig isotype controls. Finally, we initiated studies to identify the cell source of the elevated cytokines. This involved the utilization of two cell sorting techniques: magnetic activated cell sorting

(MACS) and fluorescence activated cell sorting (FACS). Thy-1$^+$ cells and later CD4$^+$ cells were initially examined to determine whether T cells and, in particular, CD4$^+$ T cells were the principal source of the elevated Th1 and Th2 cytokine gene expression detected and also to analyze when CD4$^+$ T cells first exhibited elevations in cytokine gene expression.

MATERIAL AND METHODS

Animals

BALB/c female mice were purchased from the small Animal Division of the National Institutes of Health, Bethesda, MD, and were used at age 8-12 weeks. The experiments herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare (NIH)78-23. Till now, the difference of mouse strains with respect to the immune defense against BA has not been carefully examined. From Street's *et al. in vitro* work, the T cell clones derived from BA-immunized mice showed similar cytokine production pattern, even though they were derived from several different mouse strains including Balb/c and C57BL/6 mice (11). Recently, a so-called If-1 gene has been shown to regulate some of the cytokine production in Newcastle disease virus-infected mice (76). BALB/c mice, containing If-1^l allele, exhibited low levels of IFN-γ, TNF-α and IL-6 mRNA. Whether this is true in our system needs to be tested in the future.

Antibodies

Antibodies used for staining of the cells included anti-CD4 (mAb GK1.5) (77), anti-CD8 (mAb 2.43) (78), anti-B220 (mAb 6B2) (79), anti-Thy 1 (mAb 30-H12) (80), and a blocking mAb that prevents the Fc binding (24G2, a rat IgG2b antibody specific for the mouse FcγRII and FcγRIIIα) (81). Antibodies used for the *in vivo* cytokine

intervention experiments included: anti-IFN-γ (XMG-6, a rat IgG1 that neutralizes mouse IFN-γ) (40), anti-IFNα/β (SaIFN-α/β, a polyclonal sheep antibody that neutralizes mouse IFN-α and IFN-β) (82), anti-IL-2 (S4B4, a rat IgG2a anti mouse IL-2 mAb) and the isotype control antibodies J4-1 (a rat IgG1 anti-*Nippostrongylus brasiliensis* mAb) (139), sheep IgG and GL117 (a rat IgG2a anti-β-gal mAb). The way to purify these antibodies is described below: Ab ascites fluid from nude mice was purified by $(NH_4)_2SO_4$ fractionation and ion exchange chromatography. Then they were dialyzed against 0.9%NaCl.

Immunization of Mice with *Brucella abortus* (BA)

Mice were injected i.v. with 0.2 ml of a 1:25 dilution of packed, washed BA (fixed, killed whole BA ring test antigen from USDA) in normal saline for experiments performed to study splenic cytokine gene expression, and in the foot pad with 0.05-0.1 ml of a 1:25 dilution of packed, washed BA for experiments performed to study cytokine gene expression in the draining (popliteal) lymph node.

Cell Sorting Analysis

Spleen cell suspensions from BA-immunized mice at various time points after immunization were stained with the selected conjugated antibody. The spleen cell suspensions were first incubated for 10 min with the selected biotinylated monoclonal Ab (0.2-1 $\mu g/10^6$ cells) (generously provided by Dr. F. Finkelman) in DMEM (Biofluids, Inc. Rockville, MD) containing 5% calf serum, washed and then incubated for 10 min

with FITC-avidin (2 μg/10^6 cells) (Becton Dickinson, Mountain View, CA). Single-color flow cytometric measurements were made with the FACSCAN (Becton Dickinson) equipped with the Consort 30 computer system (Becton Dickinson FACS System, Mountain View, CA). The cells were sorted either by the FACS System (FACS II, Becton Dickinson) or by a magnetic activated cell sorter (MACS, Miltenyl Biotech GmbH, Bergisch Gladbach, Germany). Three to five X 10^7 cells were used for either the FACS or the MACS sorting. To sort the cells by the MACS, the cells were further stained for 10 min with biotinylated superparamagnetic microparticles (5 μl/10^8 cells) (Miltenyl Biotech GmbH) in PBS/5mM EDTA. In the MACS sorting, the cells were dissolved in PBS/1% BSA and then passed in a steelwool matrix inserted into the field of a permanent magnet. The purity of the separated cell populations were analysed by the FACSCAN. The purifications were more than 92% pure for the positive populations and more than 95% for the negative populations in all the sorts presented. The mechanism of MACS sorting is shown in Fig. 3 (83).

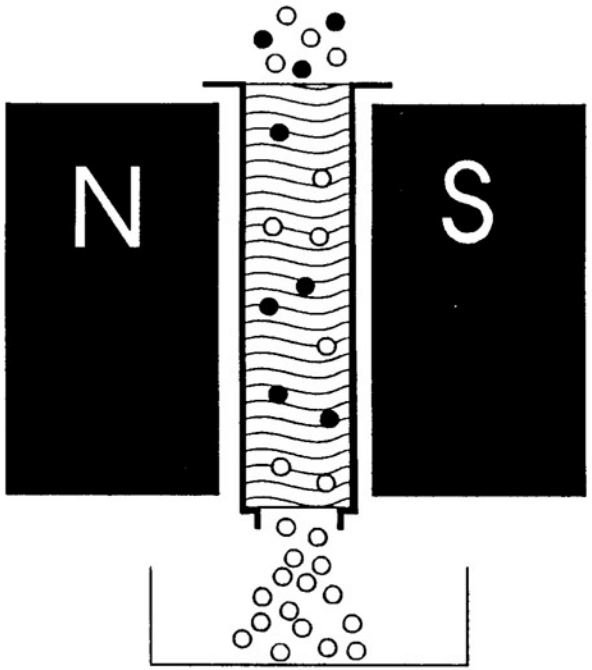
Quantitation of Cytokine mRNA

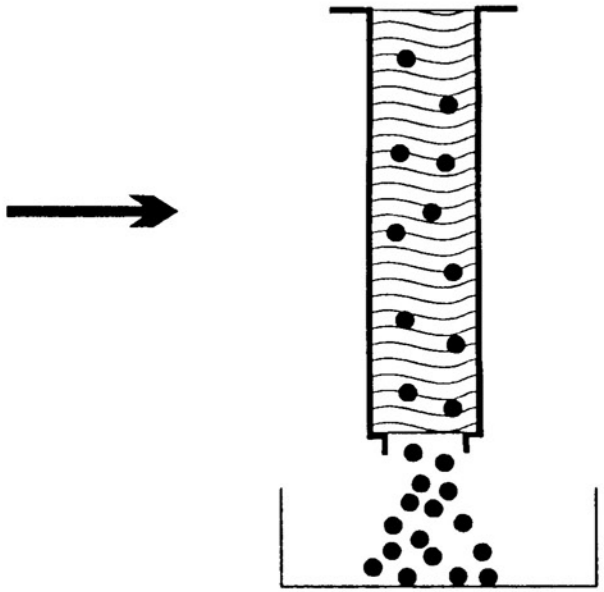
Isolation and purification of RNA

RNase-free plastic and water were used throughout the assay. Tissues were homogenized in RNAzol (Cinna/Biotecs, Friendswood, TX) at 50 mg of tissue/ml or 5x10^6 cells/ml RNAzol. Two-tenth ml of chloroform was added per 2 ml of homogenate, the samples were tightly covered, shaken vigorously for 15 seconds, and then incubated on ice for at least 5 min. The suspension was centrifuged at 12,000xg

Fig. 3. Mechanism of MACS sorting. The cells are first stained with biotinylated specific antibody, followed by FITC-avidin and finally biotinylated magnetic beads. The stained cells are then passed through a steelwool matrix inserted into the field of a magnet. In the negative selection, only the unstained cells (open circle) can pass through the matrix, while the stained cells (black dot) are attracted by the magnetic force and stay in the matrix. In the positive selection, the matrix is removed from the magnetic field and all the cells can come out of the matrix .

Magnetic Activated Cell Sorting





Negative selection

Positive selection

(4°C) for 15 min. The aqueous phase was transferred to a fresh tube, to which an equal volume of isopropanol was added. After mixing, the samples were incubated for at least 30 min at -20°C. Samples were centrifuged for 15 min at 12,000xg (4°C). The resultant RNA precipitate was transferred to a 1.2 ml centrifuge tube and washed twice with 75% ethanol. The final preparation was suspended in water and quantitated spectrophotometrically. To ascertain whether the RNA was intact and whether the concentration had been determined correctly, the purified RNA (1 μg) was electrophoresed on a 2% formaldehyde gel containing ethidium bromide (84). The gel was photographed and individual lanes were examined for the presence of the 18S and 28S ribosomal bands, the absence of RNA degradation, and whether equal loading of the RNA on each lane had occurred.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The coupled RT-PCR reaction used here was a modification of that described by Diamond *et al.* (85). Reverse transcription of RNA was performed in a 25 μl final volume containing: 1) 2.5 μl of a 10 mM mix of all four deoxynucleotide triphosphates (dNTP): 2) 5 μl of 5X reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$; 3) 2 μl 0.1 M DTT; 4) 2 μl random hexamers (0.5 U/25 μl); 5) 3 μl or 1.5 μl total RNA (1.2 μg/μl); 6) 8.7 μl double distilled H_2O. This was mixed, heated to 70°C for 5 min to denature the RNA, cooled on ice, and 1.2 μl of "Superscript" reverse transcriptase (RT from Bethesda Research Laboratories, Rockville, MD) (200 U/ml for 3.6 μg of RNA and 100 U/ml for 1.8 μg of RNA) was added. This

was again mixed, centrifuged, and incubated at 37°C for 60 min. The reaction was stopped by heating at 90°C for 5 min. then quickly cooled on ice.

The cytokine-specific primers for the polymerase chain reaction (PCR) were selected as previously described (86) and are depicted in Table I. The product amplified from cDNA could be distinguished from amplified genomic DNA, since primers were selected that amplified the intervening sequences between two exons. To the 2.5 μl or 5 μl of RT mix (containing 0.36 μg of RNA at the beginning), the following components were added: 1) 4 μl dNTP mix (10 mM); 2) 5 μl 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM $MgCl_2$, 1 mg/ml BSA); 3) 2 μl sense primer (0.2 μg/μl); 4) 2 μl anti-sense primer (0.2 μg/μl); 5) 31 μl or 33.5 μl double distilled H_2O; 6) 1 μl Taq polymerase (Promega) (1 U/μl). After an initial incubation at 95°C for 5 min, temperature cycling was initiated with each cycle as follows: 1) 95°C for 1 min (denaturation); 2) 53°C for 1 min (annealing of primers); 3) 72°C for 2 min (primer extension). For each gene product, the optimum number of cycles was determined experimentally, and was defined as that number of cycles that would achieve a detectable concentration which was well below saturation conditions.

IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9 and IL-10 were assayed. To verify that equal amounts of RNA were added in each PCR reaction within an experiment and to verify a uniform amplification process, hypoxanthine-guanine phosphoribosyl transferase (HPRT), a "house-keeping" gene cDNA, was also obtained by reverse transcriptase reaction and amplified for each assay (87).

Table I: **Primer sequences for amplification of cytokine cDNA during PCR and probe sequences for detection of amplified DNA product on Southern blot.**

Cytokine	anti-sense and sense primer	Bases Spanned	Probe	Bases spanned	No. of PCR cycles
IL-2	GAGTCAAATCCAGAACATGCC TCCACTTCAAGCTCTACAG	122-140 349-369	CTCCCCAGGATGCTCACCTTC	256-277	18
IL-4	GAATGTACCAGGAGCCATATC CTCAGTACTACGAGTAATCCA	110-130 472-493	AGGGCTTCCAAGGTGCTTCGCA	271-298	18
IL-5	GACAAGCAATGAGACACGATGAGG GAACTCTTGCAGGTAATCCAGG	129-150 342-363	GGGGGTACTGTGGAAATGCTAT	240-262	22
IL-6	TTCCATCCAGTTGCCTTCTTGG CTTCATGTACTCCAGGTAG	73-94 414-432	ACTTCACAAGTCCGGAGA	127-144	13
IL-9	GATGATTGTACCACACCGTG CCTTTGCATCTCTGTCTTCTGG	1573-1593 2873-2894	GCCTGTTTTCCATCGGGTGAAA	1648-1669	23
IL-10	CGGGAAGACAATAACTG CATTTCCGATAAGGCTTGG	147-163 315-333	GGACTGCCTTCAGCCAGGTGAAGACTTT	209-227	14
IFN-γ	AACGCTACACACTGCATCTTGG GACTTCAAAGAGTCTGAGG	73-94 291-310	GGAGGAACTGGCAAAAGGA	229-248	13
HPRT	GTTGGATACAGGCCAGACTTTGTTG GATTCAACTTGCGCTCATCTTAGGC	514-538 652-678	GTTGTTGGATATGCCCTTGAC	562-582	7

Detection of the Amplified Product by Southern Blot Analysis

10 ul of the final reaction mix was loaded in a 1% agarose gel and run at 120 volts for 1 hour. The gel was then denatured by soaking for 25 min twice in several volumes of 1.5 M NaCl, 0.5 N NaOH with constant, gentle agitation, The gel was then rinsed briefly in deionized water, and neutralized by soaking for 15 min twice in several volumes of 1.5 M NaCl, 1 M Tris (pH 7.4), at room temperature with constant agitation. Finally the gel was soaked in 20X SSPE for 30 min before the Southern transfer.

The DNA was then transferred to a nylon membrane by standard blotting procedures (84). The membrane was UV crosslinked using the UV Stratalinker 1800 (Stratagene, CA). Blots were prehybridized at 42°C for 5 hrs in a solution containing 6X SSPE, 10X Denhardt's solution, 2.5 μl/ml salmon sperm DNA (20 mg/ml) and 1% SDS, then hybridized at 49°C overnight in 6X SSPE and 1% SDS with appropriate ^{32}P-labeled probe (15 MCi/blot). The probes were specifically selected to hybridize to a portion of the amplified segment between the nucleotide sequences complementary to the primers. This ensured the identity of the amplified segment. After hybridization, blots were washed for 15 min in 6X SSPE, 0.1% SDS and then 30 sec to 2 min (depending on the different amplified DNA) in 1X SSPE, 0.1% SDS at 49°C. The resultant radioactive signal was quantitated by a phosphorimager (Molecular Dynamics, Sunnyvale, CA) which uses a phosphor screen instead of film to detect radioactive signals on the Southern blot.

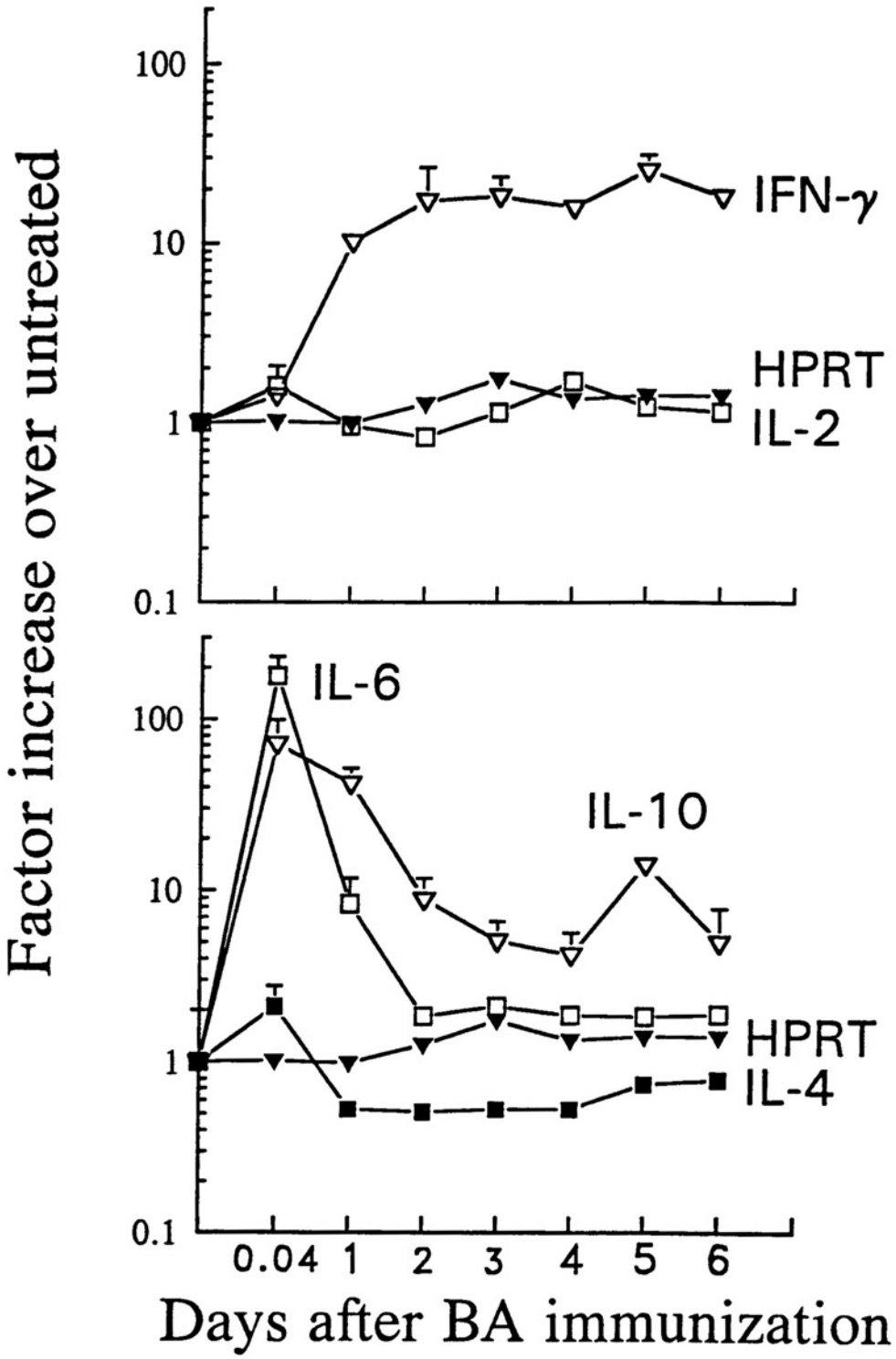
RESULTS

Expression of cytokine genes in spleen and lymph node after immunization with BA

RNA was prepared from spleens of untreated mice and from spleens of mice killed 1 hour and 1 to 7 days after BA injection. For each time point individual preparations were made from the spleens of five mice. RT was used to prepare cDNA from the RNA preparations and the PCR was used to amplify cDNA for each of the seven cytokines studied. After an appropriate number of PCR cycles, the amplified DNA was analyzed by electrophoresis, Southern blotting, hybridization with a cytokine-specific probe, and quantitative phosphoimagery as previously described (87). We have shown in previous studies (87) that the resultant signals are specific for the target cytokines and that the cycles used are well below saturation, permitting a linear relationship between input RNA and signal. Higher numbers of cycles results in a nonlinear relationship since such factors as end product inhibition and decreased enzyme activity reduce amplification of the target amplimer.

Results from the five samples for each time point are shown in Fig. 4. This experiment was repeated two times with 5 mice per time point and highly reproducible results were obtained. The mean value and standard error for each time point is shown as the factor increase over the untreated control which was arbitrarily given a value of 1. Each cytokine exhibited a specific pattern while the expression of the "house-keeping" gene, HPRT, changed little throughout the course of the primary immune response. Two cytokines, IL-6 and IL-10 were detected by 1 hour after i.v. injection of BA. In marked

Fig. 4 Kinetics of Th1/Th2 cytokine gene expression in spleens following primary BA immunization. The mean and standard error of the factor increase over the untreated control (arbitrarily given a value of 1) from the tissues of five individual mice are shown for each time point. All data were individually divided by the mean of the untreated control and log transformed prior to the calculation of the mean and standard error in order to maintain a normal distribution. The anti-logs of the means and standard errors of the factor increase over the mean of the untreated control are depicted. Reverse transcription from total RNA to cDNA was performed and cDNA was amplified with cytokine-specific primers, subjected to electrophoresis, and hybridized with specific probes by the Southern blot technique. The blots were exposed to phosphor screens and the resulting images were analyzed with a Molecular Dynamics phosphorimager. IL-5 and IL-9 did not change significantly during the course of the response (data not shown).


Factor increase over untreated
100
10
1
0.1
IFN-γ
HPRT
IL-2
IL-6
IL-10
HPRT
IL-4
0.04
1
2
3
4
5
6
Days after BA immunization

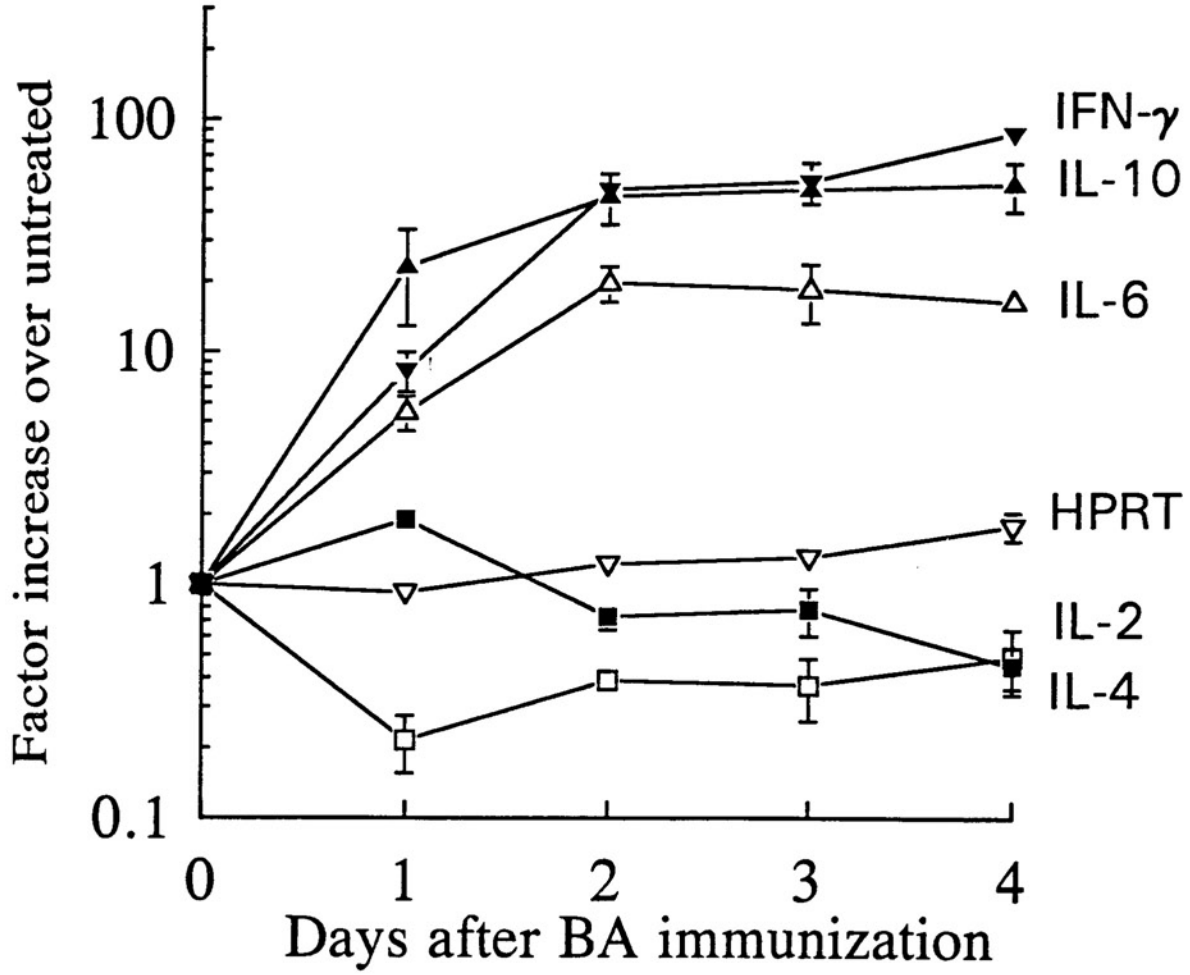
contrast, IFN-γ, which was not elevated at 1 hour, was increased greater than 10X by 1 day after injection. Both IFN-γ and IL-10 remained elevated throughout the course of the measured response. IL-6, in contrast, was decreased by 1 day after injection and from day 2 to day 7 was only slightly elevated over untreated controls. The cytokines IL-4 and IL-9 (data not shown) showed little change in gene expression during the time course examined.

As a comparison, the kinetics of cytokine gene expression was also examined in popliteal lymph nodes from mice injected in the footpad with BA. Total RNA was purified from popliteal lymph nodes 1, 2, 3 and 4 days after injection of BA into the footpad. As shown in Fig. 5, a cytokine gene expression pattern similar to that observed in the spleen was obtained, with IFN-γ, IL-10, and IL-6 being elevated by day 1, IL-2 exhibiting no increase and IL-4 some decrease. Neither IL-9 nor IL-5 was elevated during the course of the response. IL-6 gene expression in the popliteal lymph node was more prolonged than expression of the same cytokine gene in the spleen following BA immunization.

Interferons inhibit Th2 cytokine gene expression in BA

Experiments were performed to determine whether cytokine gene expression following immunization with BA was regulated by IFN-γ and/or IFN-α/β. Mice were injected i.v. with 0.1 ml of a polyclonal anti-IFN-α/β IgG (a gift of Dr. Ion Gresser, Villejuif, France) and 1.0 mg of a monoclonal anti-IFN-γ antibody (XMG-6) and one day later, were injected with BA both i.v. and in the footpad. Four days later

Fig. 5 Kinetics of Th1/Th2 cytokine gene expression in the popliteal lymph node following immunization with BA. Data were obtained and presented as described in Fig. 1. IL-5 and IL-9 did not change significantly during the course of the response (data not shown).


Factor increase over untreated
100
10
1
0.1
0
1
2
3
4
Days after BA immunization
IFN-γ
IL-10
IL-6
HPRT
IL-2
IL-4

the mice were killed and RNA was purified from the spleen and popliteal lymph node. As shown in Fig. 6, BA-injected mice treated with the anti-IFN antibodies showed a marked change in the cytokine gene expression pattern as compared to mice treated with BA and either no antibodies or control antibodies (described in M&M). In both the spleen and lymph node, IL-4, IL-5, and IL-9 showed increased gene expression over that of either control. IL-9, which was expressed at markedly higher levels in the lymph node than the spleen, was markedly reduced following BA treatment and the administration of the blocking antibodies increased IL-9 expression but did not restore the expression to that seen in the untreated controls. In contrast to these cytokines, IL-2 (data not shown) and IL-10 showed no change and IFN-γ and IL-6 gene expression were either not affected (spleen) or were decreased by this treatment (popliteal lymph node). This difference in tissues may be partly due to the higher expression of IFN-γ and IL-6 in lymph node compared to spleen at 4 days after BA injection (see Fig. 4 and Fig. 5).

Blocking anti-IL-2 antibody does not affect cytokine gene expression

The surprising lack of elevations of IL-2 cytokine gene expression following immunization with BA suggested that either constitutive IL-2 production was sufficient to support the response, that IL-2 might be regulated at the translational level, or that IL-2 production might not be necessary to support the response. To test these hypotheses, BALB/c mice were immunized with BA, both i.v. and in the footpad, in the presence of anti-IL-2 antibodies (2 mg/mouse), which in other *in vivo* immunization systems have been shown to affect immune responsiveness (88). As with the previously described

Fig. 6a Effects of the combined administration of monoclonal anti-IFN-γ (XMG-6) antibody and polyclonal anti-IFN-α/β antibody on Th1/Th2 cytokine gene expression in spleen following BA immunization i.v.. One microgram of XMG-6 and 0.1 ml. of sheep anti-mouse IFN-α/β IgG antibodies were administered one day prior to immunization with BA. Another group was treated the same except that irrelevant control antibodies, J4-1 and normal sheep IgG, were administered instead of the anti-IFN antibodies. All groups were killed at day 4 after BA immunization. All data were individually normalized to HPRT, divided by the mean of the untreated control and log transformed prior to the calculation of the mean and standard error in order to maintain a normal distribution. The anti-logs of the means and standard errors are depicted as described in Fig. 4. IL-6 and IL-2 did not change significantly between different treatment groups (data not shown).

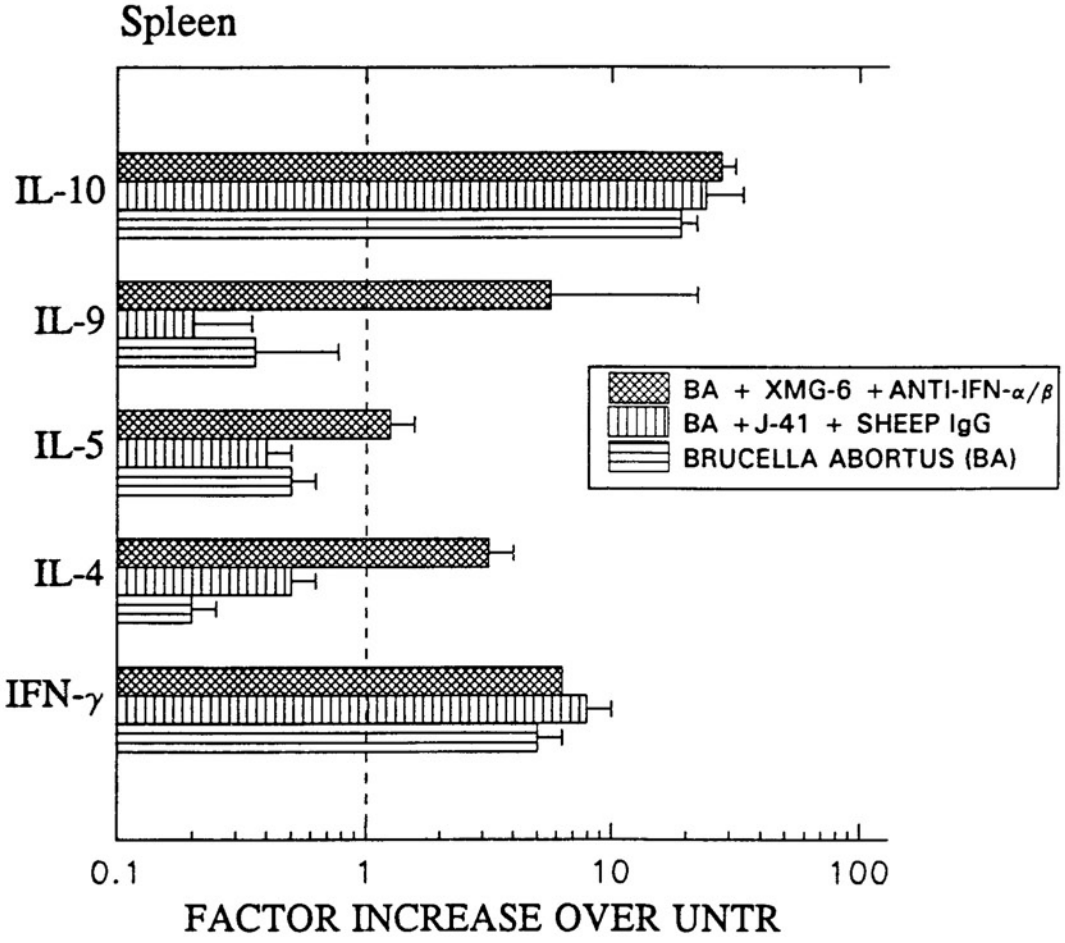
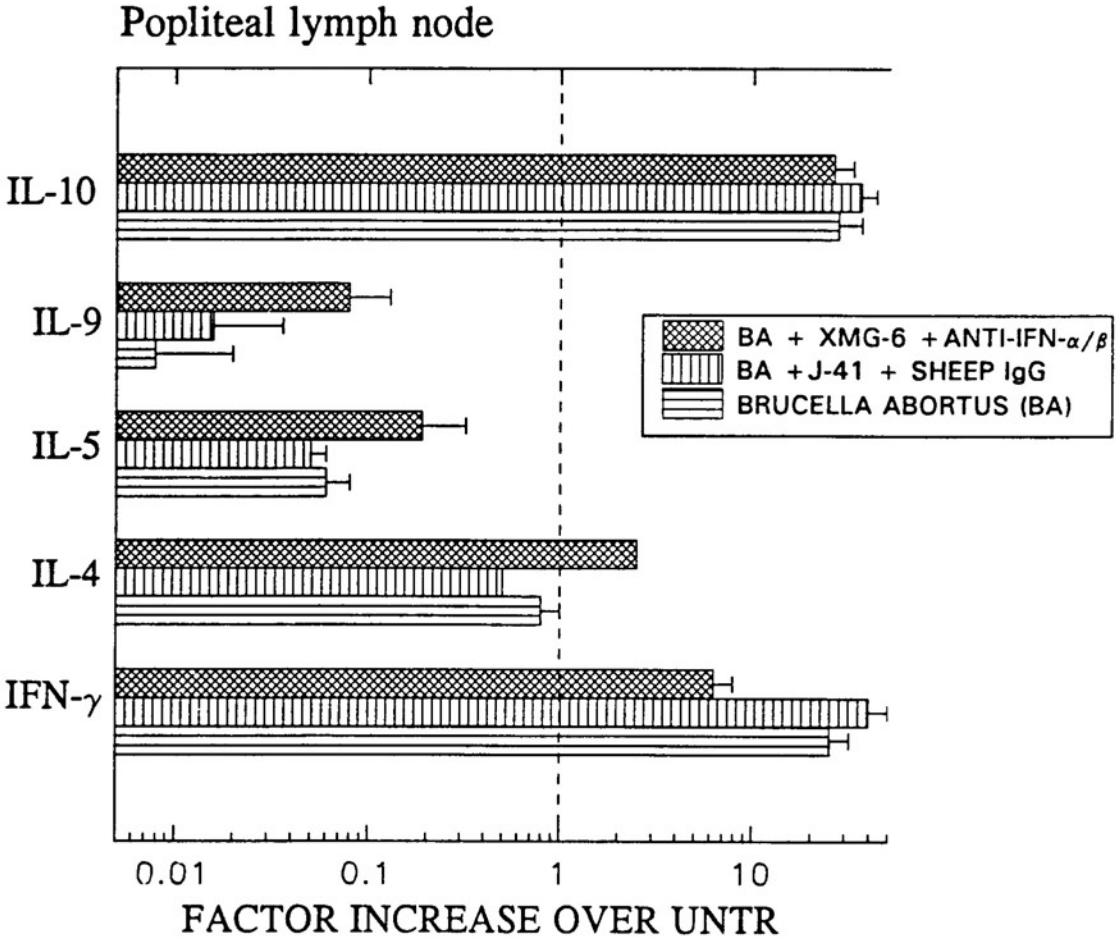

Spleen
IL-10
IL-9
IL-5
IL-4
IFN-γ
BA + XMG-6 + ANTI-IFN-α/β
BA + J-41 + SHEEP IgG
BRUCELLA ABORTUS (BA)
0.1
1
10
100
FACTOR INCREASE OVER UNTR

Fig. 6b. Effects of the combined administration of monoclonal anti-IFN-γ (XMG-6) antibody and polyclonal anti-IFN-α/β antibody on Th1/Th2 cytokine gene expression in the popliteal lymph node following BA injection in the footpad. Treatment groups were the same as those shown in Fig. 3 and the data were obtained and presented as described in Fig. 6a. IL-10 and IL-2 did not change significantly between different treatment groups (data not shown).


Popliteal lymph node
IL-10
IL-9
IL-5
IL-4
IFN-γ
BA + XMG-6 + ANTI-IFN-α/β
BA + J-41 + SHEEP IgG
BRUCELLA ABORTUS (BA)
0.01
0.1
1
10
FACTOR INCREASE OVER UNTR

intervention experiment involving the IFNs, mice were killed 4 days after immunization and RNA was purified from both spleen and lymph nodes. Neither cytokine gene expression (data shown in Fig. 7) nor immunoglobulin isotype selection was affected by this treatment (data not shown).

Cytokine gene expression by different populations of cells

Cells were sorted into specific populations at 1 hour, 1 day, and 7 days after BA immunization using both FACS and MACS. Both techniques yielded similar results. At one hour, IL-10 and IL-6 were expressed exclusively by Thy-1$^-$ cells as demonstrated in several sorts (Table II). The purity of the Thy-1-sorted cells is shown in Fig. 8. At 1 and 7 days, the cytokines that exhibited increased levels of gene expression were primarily IFN-γ and IL-10, both of which were identified as being predominantly in the Thy-1$^+$ population (Table II). In addition, in order to determine which T cell populations were actually producing these elevated cytokines, we sorted for both CD4$^+$ and CD8$^+$ cells and determined that IL-10 and IFN-γ were made by both the CD4$^+$ and CD8$^+$ populations at 1 day after BA immunization (Table III). Because both the CD4$^+$ and CD4$^-$ populations expressed IL-10 and IFN-γ at 1 day, we could not distinguish whether the elevation in cytokine gene expression detected in the unseparated cells was partially derived from the CD4$^+$ population, which represents only around 27% of the total lymphoid population, or was primarily derived from the CD4$^-$ population. To distinguish this, we sorted on the same day CD4$^+$ and CD4$^-$ spleen cells from BA-treated and control untreated mice, permitting

Fig. 7. Effects of monoclonal anti-IL-2 (S4B4) antibody administration on Th1/Th2 cytokine gene expression in the spleen following BA immunization i.v. Two micrograms of anti-IL-2 were administered one day prior to immunization with BA. Another group was treated the same except a control irrelevant antibody, Gl117, was administered instead of the anti-IL-2 antibody. All groups were killed at day 4 after BA immunization. Data were obtained and presented as described in Fig. 6a. IL-2, IL-6, and IL-9 did not change significantly between different treatment groups (data not shown).

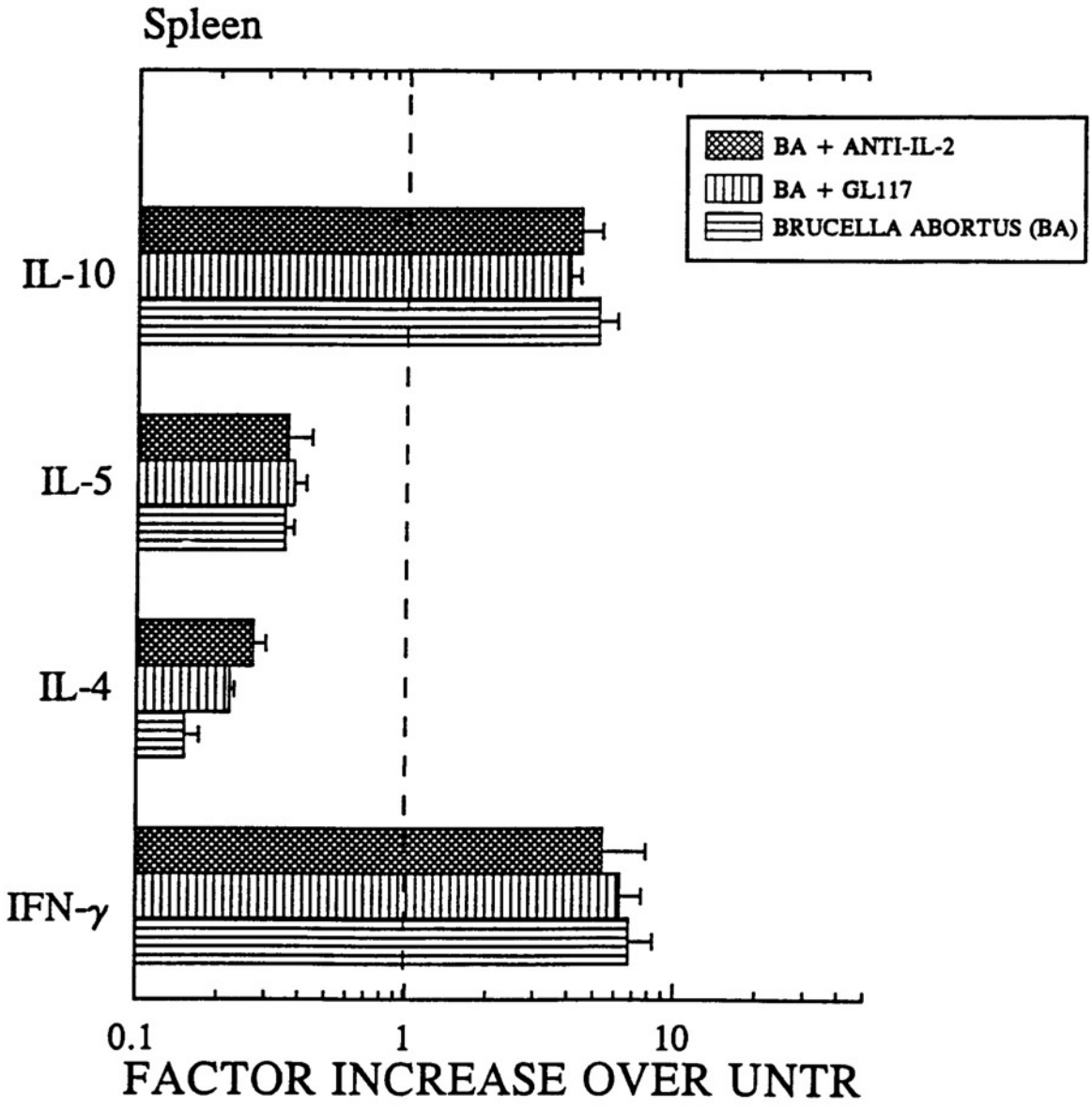

Spleen
BA + ANTI-IL-2
BA + GL117
BRUCELLA ABORTUS (BA)
IL-10
IL-5
IL-4
IFN-γ
0.1
1
10
FACTOR INCREASE OVER UNTR

Fig. 8. Flow cytometric analysis of purified Thy1$^+$ and Thy1$^-$ murine spleen cells from BALB/c mice (pools of 3) immunized for one day with BA. Thy1$^+$ spleen cells were isolated using the magnetic activated cell sorter (MACS), as described in Fig. 3 and the Materials and Methods. FACS analyses of the stained unseparated cells (A), the Thy1$^-$ population (B), and the thy1$^+$ population (C) were performed with a FACSCAN (Becton Dickinson, Mountain View, CA) and are shown as individual single histogram analyses with the percentage of cells in each of three populations (negative, dull, and bright) presented.

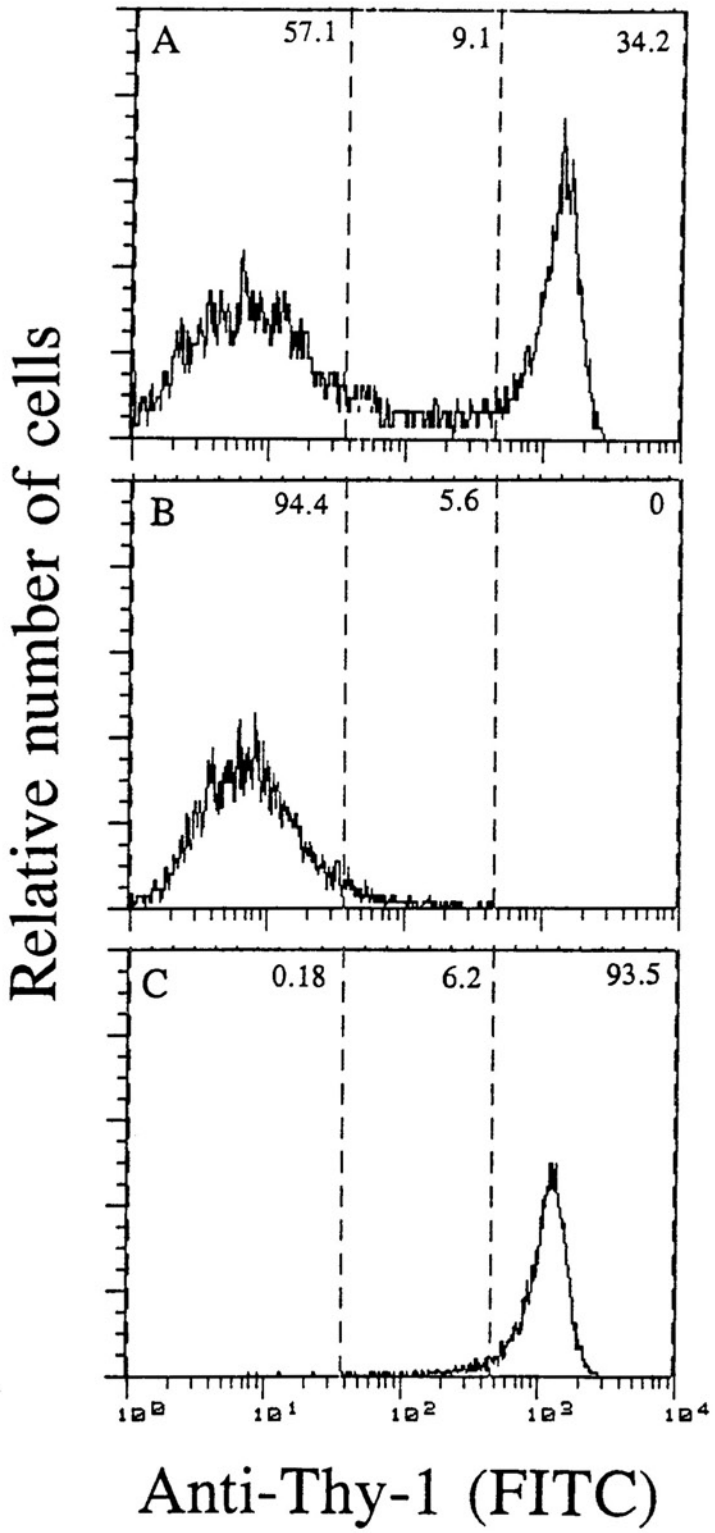

A
57.1
9.1
34.2
B
94.4
5.6
0
C
0.18
6.2
93.5
Relative number of cells
Anti-Thy-1 (FITC)

TABLE II

PERCENTAGE OF CYTOKINE GENE EXPRESSION BY THY1 SORTED CELL POPULATIONS AT VARIOUS TIME POINTS AFTER BA IMMUNIZATION[a]

CYTOKINES	1 HOUR		1 DAY		7 DAY	
	% Thy1^{+}[b]	% Thy1^{-}[b]	% Thy1^{+}[b]	% Thy1^{-}[b]	% Thy1^{+}[b]	% Thy1^{-}[b]
IL-6	0	100	------	-----	-----	----
IL-10	0	100	100	0	100	0
IFN-γ	----	----	100	0	100	0

[a]Spleen cells from BA-immunized BALB/c mice were separated into Thy1^{+} and Thy1^{-} cell populations at various time points (shown above) by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS). Pooled spleens from 3 mice were used for each sorting and the experiments were repeated twice. Cells were purified as described in the Materials and Methods and cytokine mRNA levels were measured for the sorted cell populations by RT-PCR assays as described in Fig. 1 and the Materials and Methods. The percentage of specific cytokine mRNA levels expressed by the Thy-1^{+} or Thy-1^{-} population is shown.

[b]The positive sorted cells were more than 92% pure and the negative sorted cells more than 95% pure as determined by FACS analysis.

TABLE III

PERCENT CONTRIBUTION OF CD4 & CD8 SORTED SPLEEN CELLS TO TOTAL SPLEEN IFN-γ & IL-10 mRNA LEVELS AT 1 DAY AFTER BA IMMUNIZATION[a]

CYTOKINES	PERCENT CYTOKINE GENE EXPRESSION CONTRIBUTED BY SPECIFIC SORTED CELL POPULATIONS			
	$CD4^{+}$ [b, c] CELLS	$CD4^{-}$ [b, c] CELLS	$CD8^{+}$ [b, c] CELLS	$CD8^{-}$ [b, c] CELLS
IFN-γ	39[d]/20[e]	61/80	18/2.5	82/97.5
IL-10	41/21	59/ 79	43/7.7	57/ 97.3

[a]Spleen cells from BA-immunized Balb/c mice were separated into $CD4^{+}$, $CD4^{-}$, $CD8^{+}$, and $CD8^{-}$ cell populations at 1 day after BA immunization (shown above) by magnetic activated cell sorting (MACS) and fluorescence activated cell sorting (FACS). Pooled spleens from 3 mice were used for each sorting and the experiments were repeated twice. Cells were purified as described in the Materials and Methods and cytokine mRNA levels were measured for the sorted cell populations by RT-PCR assays as described in Fig. 1 and the Materials and Methods.

[b]The positive sorted cells were more than 92% pure and the negative sorted cells more than 95% pure as determined by FACS analysis.

[c]The percentage of $CD4^{+}$ cells in spleen was 27% after 1 day BA-treatment. 73% for $CD4^{-}$ cells, 12% for $CD8^{+}$ and 88% for $CD8^{-}$ cells.

[d] An equal quantity of RNA was analyzed for cytokine expression from either the positive or negative sorted population in a given experiment. The resultant signals were summed and the percent contributed by each population depicted..

[e] The calculated percent contribution by a specific cell population to the total expression in the spleen. This value was derived by factoring in the percent of each cell population in the total spleen. These values are based on the assumption that different cells have an equal quantity of RNA and that the ratio of mRNA/rRNA is also equal.

the direct comparison of $CD4^+$ cells from the immunized and unimmunized populations. The purity of the CD4 sorted cells was shown in Fig. 9. As shown in Fig. 10 and table IV, IL-10 but not IFN-γ was elevated in the $CD4^+$ BA-treated spleen cells compared to the $CD4^+$ untreated spleen cells. An important caveat is our observation that IFN-γ was increased in cells stored in sorting media for extended periods (Fig. 10). As expected, no significant change in IL-2 cytokine gene expression was detected between the $CD4^+$ BA-treated and untreated spleen cells.

Fig. 9a. Flow cytometric analysis of purified $CD4^+$ and $CD4^-$ murine spleen cells from untreated BALB/c mice (pools of 3) isolated using the magnetic activated cell sorter (MACS). Cell suspensions (pools of 3) were stained with biotinylated anti-CD4 antibody and separated as described in Fig. 3 and the Materials and Methods. FACS analyses of the stained unseparated cells (A), the $CD4^-$ population (B), and the $CD4^+$ population (C) were performed with a FACSCAN (Becton Dickinson, Mountain View, CA) and are shown as individual single histogram analyses with the percentage of cells in each of three populations (negative, dull, and bright) presented.

UNTREATED MICE

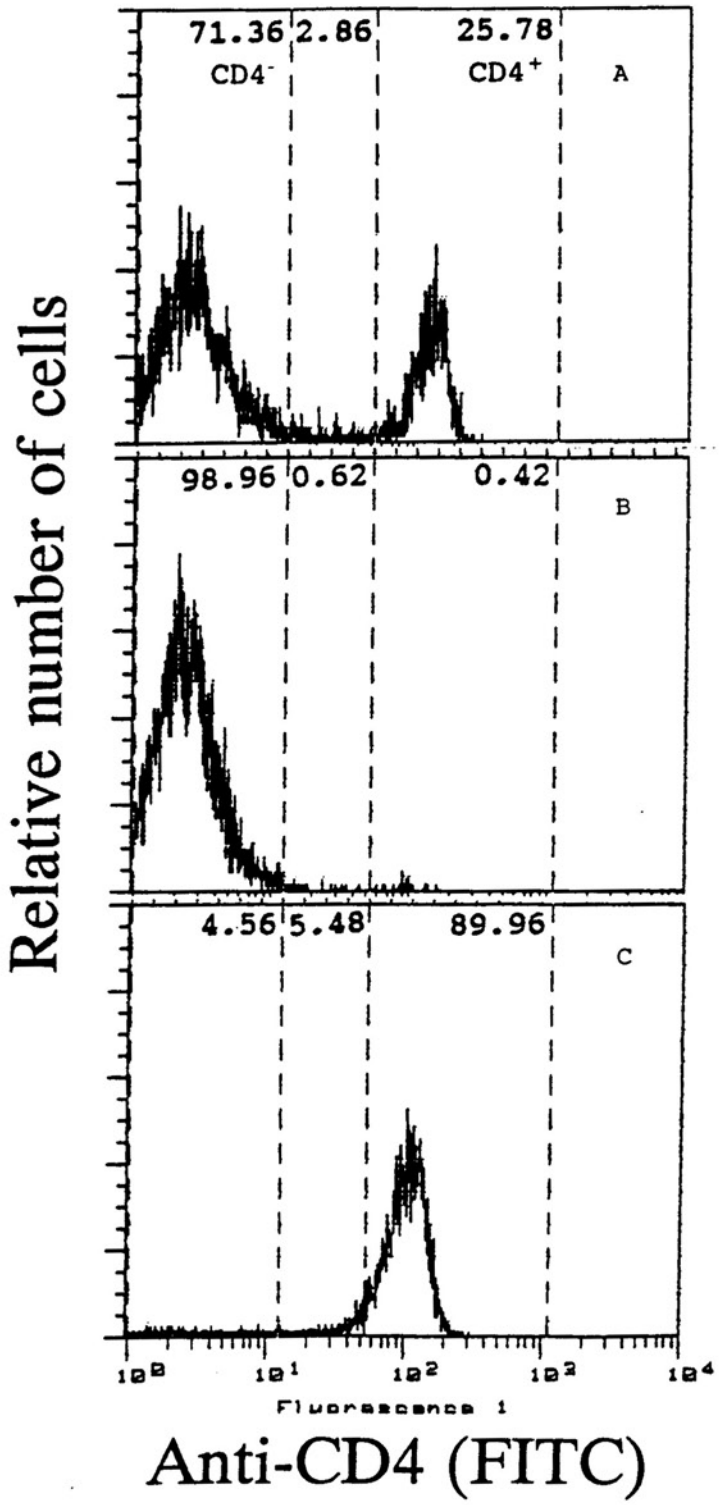


Fig. 9b. Flow cytometric analysis of purified $CD4^+$ and $CD4^-$ murine spleen cells from BALB/c mice (pools of 3) immunized for one day with BA. $CD4^+$ spleen cells were isolated using the magnetic activated cell sorter (MACS), as described in Fig. 3 and the Materials and Methods. FACS analyses of the stained unseparated cells (A), the $CD4^-$ population (B), and the $CD4^+$ population (C) were performed with a FACSCAN (Becton Dickinson, Mountain View, CA) and are shown as individual single histogram analyses with the percentage of cells in each of three populations (negative, dull, and bright) presented.

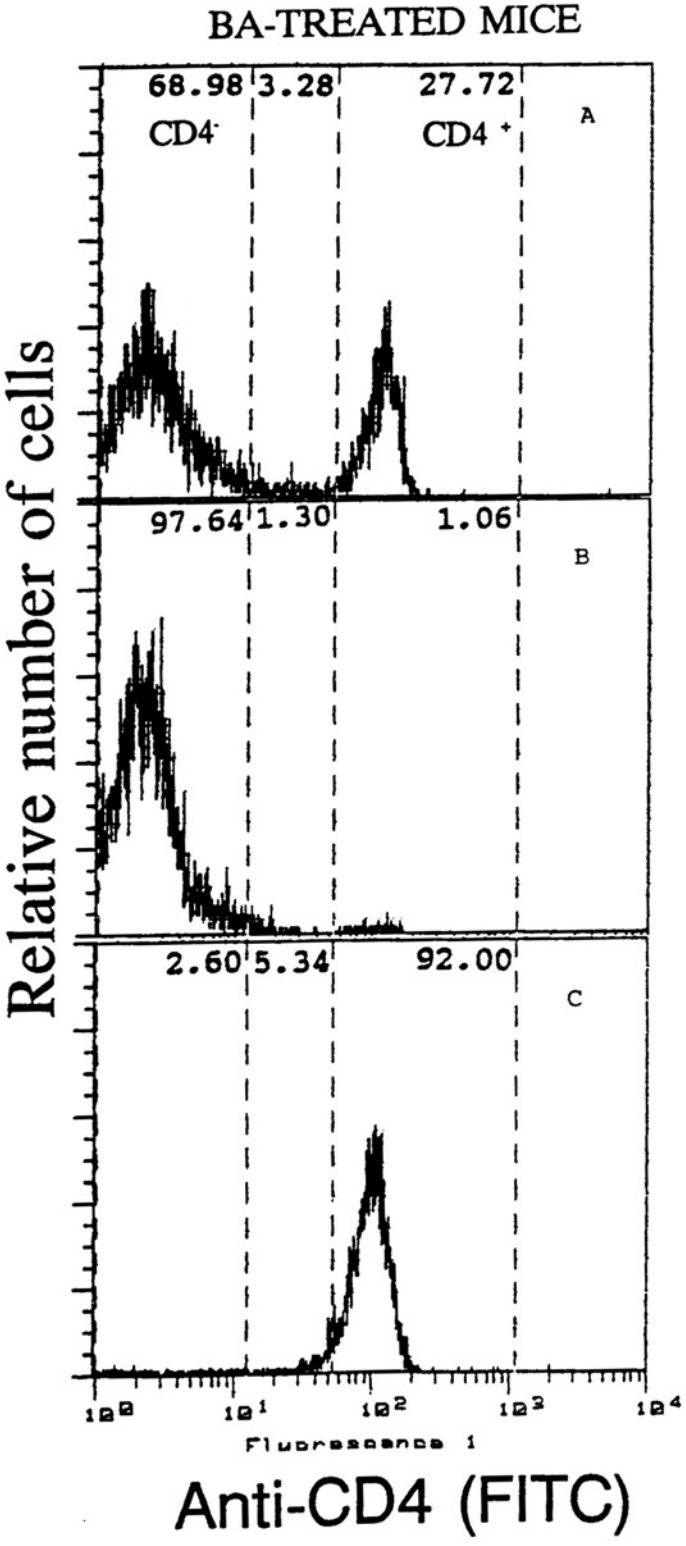

BA-TREATED MICE
68.98
3.28
27.72
CD4-
CD4+
A
97.64
1.30
1.06
B
2.60
5.34
92.00
C
Relative number of cells
Anti-CD4 (FITC)

Figure 10. Cytokine mRNA levels in $CD4^+$ and $CD4^-$ sorted spleen cells from untreated mice and mice immunized with BA for 24 hours. Single cell suspensions were prepared from whole spleen (3 animals per treatment group) and stained with biotinylated anti-CD4 antibody (GK1.5) followed by FITC-avidin and then biotinylated magnetic beads. The cells were then passed over the magnetic activated cell sorter (MACS) and $CD4^+$ and $CD4^-$ cells were isolated as described in the Materials and Methods. As described in Fig. 1 and the Materials and Methods, specific cytokine gene expression was analyzed by the quantitative RT-PCR assay. (N2) means the spleen cells frozen by liquid nitrogen immediately after removed from the mice. (STAINED) means the total spleen cells that were stained by the antibody, avidin and the beads.

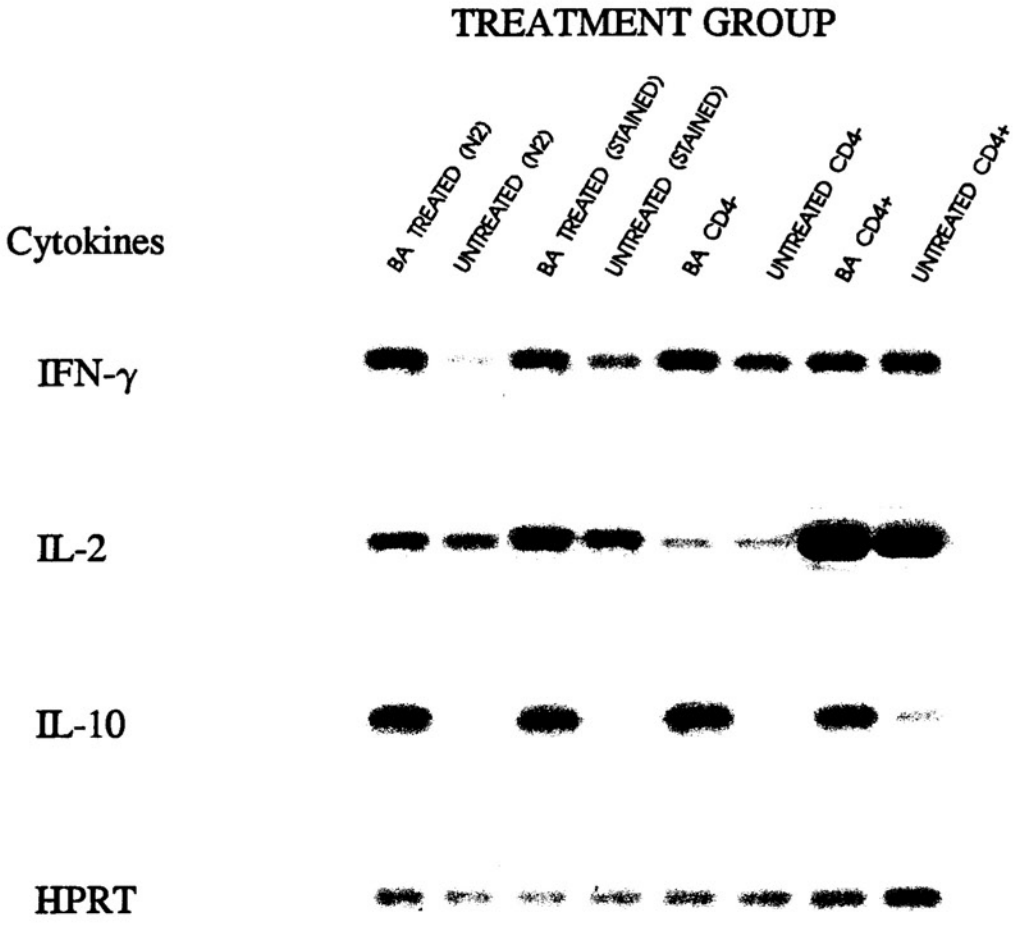

TREATMENT GROUP
BA TREATED (N2)
UNTREATED (N2)
BA TREATED (STAINED)
UNTREATED (STAINED)
BA CD4-
UNTREATED CD4-
BA CD4+
UNTREATED CD4+
Cytokines
IFN-γ
IL-2
IL-10
HPRT

TABLE IV

CYTOKINE mRNA LEVELS IN CD4-SORTED SPLEEN CELLS

FROM UNTREATED MICE AND 1 DAY-BA-TREATED MICE[a]

cytokines	TREATMENT GROUP							
	BA treated (N2)[b]	untreated (N2)[b]	BA treated/ stained[c]	untreated/ stained[c]	BA $CD4^-$	untreated $CD4^-$	BA $CD4^+$	untreated $CD4^+$
IFN-γ	383	48	513	111	515	175	283	182
IL-2	263	135	1069	373	66.7	60	6743	2171
IL-10	290	12.5	408	9.6	468.5	4	322	34

[a] Quantitation of cytokine-specific message depicted in Figure 11. Quantitation of Southern blots using the phosphorimager (Molecular Dynamics) is as described in the Materials and Methods. All values were normalized to HPRT.

[b] (N2) refers to samples of tissue homogenized in RNAzol and snap-frozen in liquid nitrogen. RNA was purified and RNA-specific cytokine message reverse-transcribed and amplified as described in the Materials and Methods.

[c] Prior to cell sorting, samples from cell suspensions stained with specific antibody were pelleted and resuspended in RNAzol. RNA was purified and analyzed for specific cytokine message as described in the Materials and Methods.

DISCUSSION

These studies established a novel pattern of elevated cytokine gene expression *in vivo* following immunization with BA, as compared with that to another immunogen. This response was characterized by simultaneous elevation of IL-10 and IFN-γ from days 1 to 7 after immunization. IFNs were the key cytokines integral in the establishment of this specific immune response, since administration of blocking antibodies to IFNs increased expression of the Th2 cytokines, IL-4, IL-5, and IL-9, and altered Ig isotype production (personal communication with Dr. Fred Finkelman). $CD4^+$ cells were activated by 1 day after immunization, expressing elevated IL-10, but not increased IL-2 gene expression. Furthermore, administration of blocking anti-IL-2 antibodies affected neither cytokine gene expression nor immunoglobulin isotype production, suggesting that this cytokine was not required for the initiation of T cell responsiveness or the progression of this primary humoral immune response.

These data suggest that during the primary immune response both the specific cytokine produced and its temporal pattern of expression can be highly controlled. As with the Th1 and the Th2 responses observed at later stages of an immune challenge, a distinctive cytokine pattern rapidly develops following primary immunization. This cytokine gene expression pattern was highly reproducible with little variation from mouse to mouse. Recent studies of cytokine gene expression following infection with the nematode parasite, *Heligmosomoides polygyrus*, have in fact determined a markedly different response with elevations of IL-4 and IL-5 and suppression of IFN-γ (Svetic',

et al., submitted). Analysis of the *in vivo* primary immune response to goat anti-mouse IgD demonstrated a pattern of cytokine gene expression where $CD4^+$ T cells first exhibited elevated IL-2, with peak expression by day 3 after immunization. Subsequently, by days 4-6 after immunization, both Th1 and Th2 cytokines became elevated (87). *In vivo* studies of *Leishmania* have also suggested that a specific cytokine pattern can manifest itself early in the primary immune response (22,89). Taken together these data suggest that cytokine production during the primary immune response may be highly specific and determined by the particular antigen involved.

An important caveat, however, is that cytokine gene expression may not always correlate with protein production. In fact, recent data from our laboratory where we have analyzed IFN-γ protein secretion using an ELIspot assay (Gause , W.C. and Lu, P., unpublished) has shown some discrepancy with IFN-γ gene expression. Whereas increased IFN-γ gene expression was marked by 1 day after BA immunization elevated secretion was not detected until day 2 and did not reach peak levels until day 3-5. Considerable investigation will be required to determine if this difference is merely representative of the lag time between increased message and protein or whether some regulatory process occurs at the translation level.

The observed pattern of cytokine gene expression was to a large extent dependent on IFN production, since the results (Fig. 6a and 6b) demonstrated that administration of anti-IFN blocking antibodies increased expression of all the Th2-like cytokines, with the exception of IL-10. These data are consistent with previous studies where anti-IFN-γ antibody treatment suppressed IgG2a production and enhanced IgG1 (11). It is likely that

the increased ratio of IL-4/IFN-γ favored this change in isotype production, as has been shown in other systems (69). The observation that $CD4^-$, Thy-1^+ cells were the primary source of IFN-γ production suggests that $CD8^+$ T cells, natural killer cells or TCR-γ/δ cells may initially produce this cytokine. All three cell lineages, including natural killer cells (Ortaldo, pers. comm.) can express Thy-1 and also cytokines, particularly IFN-γ (67,90). Current studies are attempting to distinguish these possibilities. The time, after BA immunization, that $CD4^+$ cells first express IFN-γ is also being investigated.

The simultaneous elevation of IL-10 and IFN-γ and the lack of effect of anti-IFN-γ antibody on IL-10 gene expression was surprising since previous studies have suggested that IFN-γ downregulates IL-10 production and vice versa (17,91). These data suggest that IL-10 is regulated differently than the other Th2 cytokines and suggest it may play a unique function in IFN-mediated responses. Since IL-10 has been shown to downregulate IFN-γ production in *in vitro* studies (17), it is possible that it acts to regulate the IFN-γ-mediated response perhaps serving to dampen excessive cell-mediated immunity.

The lack of increased IL-2 cytokine gene expression in the spleen was unexpected. It was hypothesized that the considerable constitutive IL-2 expression by B cells, as has been previously shown (92), might mask slight but physiologically important changes. However, although the popliteal lymph node has a much higher T/B cell ratio, still no change in IL-2 cytokine gene expression was detected. Furthermore, comparison of sorted $CD4^+$ spleen cells from BA-treated mice with $CD4^+$ cells from untreated mice showed no increase in expression. To test whether constitutive IL-2 protein levels were

necessary to support the BA response or whether physiologically relevant increases in IL-2 protein might be secreted despite little change in IL-2 gene expression, mice immunized with BA were simultaneously administered anti-IL-2 blocking antibody, which had previously been shown to have marked effects in other *in vivo* systems (88). Consistent with the gene expression analyses, no affect of the anti-IL-2 antibody on either cytokine gene expression or immunoglobulin isotype production was detected, suggesting that this cytokine may not play an important role in the primary immune response to BA. However, it is important to consider the possibility that the dose of blocking antibody used or alternatively its method of administration may not have been optimal for blocking in this system.

No typical Thp, Th0, Th1, or Th2 patterns were observed following immunization with BA, although $CD4^+$ T cells first exhibited increased cytokine gene expression as early as 1 day after BA injection i.v. The *in vitro* studies by Street *et al.* (43) demonstrated an exclusively Th1-type immune response against BA, although they did not measure changes in IL-10 production. Other *in vitro* studies in which lectins or anti-CD3 antibody were used to activate T cells found IL-2 gene expression preceded that of IL-10 and IFN-γ (49,93-95). Similarly, *in vivo* primary immune responses to GaMδ (87) or picryl chloride (96) have also documented initial increases in IL-2 prior to elevations of other T cell derived cytokines. In contrast, injection of mice with anti-CD3 antibody *in vivo* induces pronounced elevations in IL-2, IL-4, and IFN-γ gene expression within 1 hour of antibody injection (97). Although the injection of picryl chloride or GaMδ antibody probably stimulates virgin T cells, anti-CD3 antibody could also stimulate

preactivated and memory T cells already committed to producing either Th1 or Th2 cytokines. However, anti-CD3 is a potent T cell activator and the intensity of the T cell response may be sufficient to stimulate virgin T cells to differentiate into IL-4- and IFN-γ-secreting cells.

The T cell-mediated response to BA is probably also a result of naive T cell activation, since it is unlikely that mice have been consistently exposed to BA antigens previously and the response kinetics showed little variation from mouse to mouse. In contrast to the generalized cytokine gene expression pattern observed following anti-CD3 injection, the BA response was highly specific with elevations detected primarily for IL-10 and IFN-γ, and little change being detected in IL-2, IL-4, IL-5, or IL-9. These data suggest that the immune response elicited by BA is sufficiently strong to promote the development of a specific T cell derived cytokine pattern as early as 1 day after immunization and in the absence of an initial IL-2 increase. However, BA-mediated T cell stimulation results in a more directed pattern of T cell activation than that of anti-CD3 antibody stimulation.

Prior to the detection of elevated cytokine gene expression by T cells, marked elevations in both IL-6 and IL-10 were observed. The cell source, currently being investigated, was probably B cells and/or macrophages. This probably results from direct stimulation of these antigen presenting cells by ligands. An example would be lipopolysaccharide, which is a common constituent of gram-negative bacteria and has been shown previously to stimulate specific cytokine production by APCs (98). It has been suggested that the co-stimulator activity of APCs that is essential for the clonal

expansion of T cell-mediated immunity may be regulated by such ligands (52). The co-stimulator activity may include expression of specific cell surface adhesion molecules and the secretion of specific cytokines by a particular APC. IL-10 and IL-6 may contribute to the co-stimulatory signals that occur during antigen presentation, differentially affecting T cell cytokine production. The type of APC may also be important. For example, Radbruch and Schmitz (53) have recently demonstrated that presentation of antigen by macrophages as compared with B cells differentially affects T cell proliferation and T cell-derived cytokine production. Our data suggest that IFNs play an important role in affecting the T cell cytokine production pattern by downregulating the expression of particular Th2 cytokines.

Overall, these data suggest that during the primary immune response both the specific cytokine expressed and its temporal pattern of expression can be highly controlled. As with the Th1 and the Th2 responses observed at later stages of an immune challenge, a distinctive cytokine pattern occurs which is specific for the particular pathogen involved. It will be important in future studies to further characterize the specific cell populations associated with this response and to determine the signals characteristic to a particular pathogen that elicit a specific immune response.

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